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(57) Abstract

A rapid, sensitive and specific immunometric assay system is provided for determining the presence and/or concentration of antigens associated with infection of cereal plants by the fungal pathogen *Pseudocercosporella herpotrichoides* including a grinding apparatus for extracting such antigens in suitable liquid media. A polyclonal, monospecific antibody reagent is employed in a "forward-sandwich" immunoassay for detection of polyvalent fungal antigens associated with the development of cereal eyespot disease.

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TITLE

IMMUNOASSAY TO DETECT PSEUDOCERCOSPORELLA ANTIGEN IN CEREAL PLANTS

CROSS-REFERENCE TO RELATED APPLICATION

This application is a continuation-in-part of copending application Serial No. 07/398,265 filed August 24, 1989.

Technical Field

This invention relates to a highly sensitive and specific method for detecting polyvalent antigens in plant tissue. In particular, it relates to a rapid immunoassay for antigen associated with fungal infection of wheat causing cereal eyespot disease. Another aspect of this invention relates to a novel grinding apparatus for extraction of antigens from infected plant tissue.

Background of the Invention

Cereal eyespot (footrot) is a fungal disease of wheat, rye, oats, barley, and grasses. The disease is found in many parts of the world with cool, moist climates and is especially prevalent in the Great Lakes and Pacific Northwest regions of the United States and in portions of South America, Europe, New Zealand, Australia, and Africa. The disease cycle begins in autumn or early spring when Pseudocercosporella herpotrichoides spores carried over on field stubble from the previous year's crop are deposited on the basal culm (foot) of the young cereal plants by rain splash. The spores germinate and superficially infect outer leaf sheaths. If

cool, moist conditions persist through the winter and spring, the fungus penetrates into inner leaf sheaths and eventually reaches the stem. The resulting lesion disrupts the flow of nutrients to the upper portions of the plant and may weaken the stem sufficiently to cause the stem to be beaten down easily or "lodged". The disease may kill the plant outright, but more often results in reduced yields. Yield reduction may result from poor head filling, reduced seed numbers, or harvesting difficulties due to lodging.

The presumptive diagnosis of cereal eyespot disease primarily relies on the identification of characteristic elliptical or "eye" shaped lesions. Early in the season, lesions are found only on the outer leaf sheaths and are very easily confused with symptoms associated with other fungal cereal diseases. Other foot diseases with similar symptoms are "sharp eyespot" caused by Rhizoctonia solani (cerealis), "take-all" caused by Gaeumannomyces graminis, and "root rot" caused by Fusarium species. Even late in the season when eyespot lesions are fully developed, disease diagnosis is relatively difficult. Confirmation of eyespot usually results from a direct microscopic examination of the lesion for the presence of P. herpotrichoides spores. This technique is labor intensive and is not a reliable method of confirming the disease.

Eyespot is the only early season foot disease of wheat that usually warrants the application of fungicide for control. While severe take-all and sharp eyespot outbreaks may result in economically important crop loss, the cost benefit ratio does not usually justify treatment. Benzimidazoles and certain sterol-biosynthesis-inhibiting fungicides

have been shown to be effective treatments for cereal eyespot. Field data indicate that there is a two to three week application window when fungicide treatment results in maximum disease control. The timing of this narrow application window depends on weather conditions, cereal cultivar, and disease pressure.

If eyespot is present in the field but symptoms have not developed before the application window, growers must make decisions to use fungicides without knowing whether or not the disease is present. For these reasons, a method to detect presymptomatically the fungus associated with eyespot would result in fewer, more effective fungicide treatments.

Many assays which diagnose disease and quantitatively determine pathogen levels in animals are based on immunological techniques. These methods rely on the highly specific interaction of antibody molecules with substances, referred to as antigens, used to elicit antibody production. When an antigen is introduced into the body of a vertebrate animal, the animal's immune system reacts by generating antibodies which bind to the antigen at immunogenic sites. Large antigen molecules such as proteins and polysaccharides typically contain many different immunogenic sites. Antibody molecules produced by a single antibody-producing cell are identical and are referred to as "monoclonal" antibodies. The antibody response to most antigens is "polyclonal" in that many different antibody molecules are produced in response to each of the immunogenic sites recognized by the host. The antibody response to a given antigen is determined genetically and varies between species of animals.

Immunoassays are particularly well suited for detection of polyvalent antigens that can complex with two or more antibodies at the same time. One such assay is referred to as a "sandwich" assay since the antigen has two antibodies bound to its surface at different sites. Unlabelled antibody (capture antibody) immobilized on the surface of an insoluble solid support matrix captures the antigen contained within a fluid medium. After suitable incubation, the solid support matrix is washed to remove the fluid sample, including any unreacted antigen, and is then contacted with a solution containing a known quantity of labelled second antibody. The second antibody may be "labelled" with a variety of substances including radioisotopes, fluorescent chromophores, or enzymes. During subsequent incubation the labelled second antibody reacts with the antigen fixed to the solid support matrix and in so doing also becomes fixed. The solid support matrix is washed a second time to remove unreacted second antibody. The presence of antigen within the test sample is demonstrated by detection of the bound label. An assay based on this technique for detection of the antigen associated with serum hepatitis is described in U.S. Pat. No. 3,867,517, and is referred to as a "forward-sandwich" immunoassay. Variations of the assay format exist and include: (1) reaction of the antigen sample with the labelled second antibody before incubation with the immobilized unlabelled antibody (referred to as a "reverse-sandwich" immunoassay), and (2) incubation of antigen with labelled and unlabelled antibody at the same time ("simultaneous-sandwich" immunoassay).

Application of immunoassay technology to the field of plant pathology has been demonstrated by the work of Clark and Adams [J. Gen. Virol., 34, 475-483 (1977)]. The authors describe the use of a forward-sandwich enzyme immunoassay method to detect viral antigen in plant tissue. Methods are described to extract viral antigens from plant materials, immunize animals, purify polyclonal antibody reagents, and assay for the presence of viral antigen. The assay was both sensitive and specific for detecting viral antigens; however, the method suffers from several deficiencies. For instance, the authors utilized a relatively complex sample extraction buffer, large sample volumes, high (30°), low (4°), and room temperature incubations, and required 18-30 hours to complete. Despite its weaknesses, this assay method has served as a model for workers trying to develop immunoassays for plant pathogenic fungi.

Bolik et al. [Z. Pflanzenkr. Pflanzenschutz, 94, 449-456 (1987)] have reported development of an immunoassay for detection of P. herpotrichoides in artificially inoculated wheat stems. Rabbits were immunized with suspensions of freeze-dried mycelia. The resulting polyclonal antibodies were used to develop an immunoassay according to the method of Clark and Adams (supra). The assay detected both wheat and rye isolates of P. herpotrichoides but cross-reacted with other genera of important wheat pathogens and exhibited nonspecific reactions. Bolik et al. suggest that monoclonal antibodies be used instead of polyclonal reagents to overcome these problems.

A second immunoassay for detection of P. herpotrichoides in wheat was reported by Unger and

Wolf [J. Phytopath., 122, 281-286 (1988)]. Rabbits were immunized with ground mycelia and purified polyclonal antibodies were used to detect fungal antigens in plant extracts directly adsorbed to the surface of polystyrene plates. A complicated assay was developed that required 22 hours to complete and used three different incubation temperatures (37°, 6°, and room temperature). The authors reported reactivity to nineteen Pseudocercosporella isolates and no cross-reactivity to twelve other wheat pathogens. However, the inherent low sensitivity associated with this assay format limits the value of the results. It generally is believed that the low sensitivity of this format results from adsorption to the solid support of nonspecific substances in the sample other than the antigens of interest.

Efforts to develop immunological diagnostic assays for fungal plant pathogens were reviewed by Dewey [J. Phytopath., 122, 281-286 (1988)]. The author emphasizes that lack of progress in this area reflects the difficulty of raising specific antisera that are specific and suggests the use of monoclonal antibodies (Mab) as a solution in this regard. Dewey refers to a Mab-based immunoassay for detection of Pseudocercosporella, but makes no specific claims concerning this assay.

At this time there exists a clear need for a sensitive and specific assay to determine the presence and/or concentration of P. herpotrichoides that is also inexpensive, rapid, simple to perform, and capable of processing large numbers of plant samples. Furthermore, a method is needed that detects P. herpotrichoides before eyespot symptoms appear, at a time when fungicide application is most effective. The method also should distinguish

P. herpotrichoides from other stem disease organisms whose symptoms can easily be mistaken for eyespot. Finally, there exists a need for a suitable grinding apparatus for the extraction of antigens from infected plants.

Summary of the Invention

Applicants' invention is in part a grinding device, comprising a cylindrical tube, one end of the tube being closed with a circular base. The base of the tube having on its interior face a plurality of radiating ridges constituting a first grinding surface. The grinding device also includes a plunger having a first end and a second end, the first end being cylindrical and having radiating ridges complementary to the ridges of the first grinding surface constituting a second grinding surface. The first end of the plunger is slidably insertable into the cylindrical tube and axially rotatable therein. The second end of the plunger has a means for rotating the plunger within the cylindrical tube so as to grind material positioned between the first and second grinding surfaces. The space between the inner surface of the tube and the first end of the plunger is such that ground material escapes from the first and second grinding surfaces along the inner surface of the tube but unground material does not escape from the first and second grinding surfaces. Applicants' invention also includes a method for assaying the fungus Pseudocercosporella, in which a fungal antigen is extracted from plant tissue into an aqueous medium and a reaction system is formed on a solid support by contacting the aqueous medium containing the fungal antigen with a number of components. These components include

(1) a high titer polyclonal capture antibody monospecific for the fungal antigen;
(2) a polyclonal antibody that reacts to the antigen;
(3) a reporter mechanism.

The components are combined in proportions such that a signal from the reporter mechanism is related to the presence of, or concentration of, fungal antigen initially present in the aqueous medium. The method also includes measuring the signal from the reaction system and relating the signal to the presence or concentration of fungal antigen intitially present in the aqueous medium. Applicants' invention further includes a kit for assaying for the Pseudocercosporella antigen in cereal plants. The kit includes the grinding device and the components of the assay previously mentioned.

Brief Description of the Drawings

Figure 1 is a schematic diagram showing components of the Pseudocercosporella assay.

Figure 2a is a graph demonstrating the relationship between disease severity and Pseudocercosporella immunoassay results using infected wheat plants that were greenhouse grown.

Figure 2b is a graph demonstrating the sensitivity of the Pseudocercosporella immunoassay using samples of wheat plants infected in the field with P. herpotrichoides.

Figure 3 is a bar graph showing the cross-reactivity of a forward sandwich enzyme immunoassay performed on fungal extracts of Septoria, Gaeumannomyces and Fusarium using cross-reactive, polyclonal, rabbit-anti-P. herpotrichoides immunoglobulin (Ig) as the capture antibody.

Figure 4 is a bar graph showing the results of absorbed, monospecific rabbit-anti-Pseudocercosporella Ig used as capture antibody in a forward sandwich enzyme immunoassay and tested for cross-reactivity against fungal extracts of Septoria, Gaeumannomyces, and Fusarium antigens.

Figure 5 displays data showing that the assay is sensitive enough to detect picogram quantities of the P. herpotrichoides antigen.

Figure 6 demonstrates the utility of the tissue grinding apparatus used in conjunction with the Pseudocercosporella immunoassay.

Figure 7 shows an elevation view of a grinder tube and lid.

Figure 8 shows the top of the grinder tube base which forms the first grinding surface.

Figure 9 shows the bottom of the grinder tube base.

Figure 10 shows an elevation view of the plunger.

Figure 11 shows the top of the plunger including means for manual or automatic rotation.

Figure 12 shows the bottom of the plunger which forms the second grinding surface.

Figure 13 shows a vertical section of the assembled parts of the grinding device.

Figure 14 shows a vertical section of a preferred embodiment of the grinding device.

Detailed Description of the Invention

The present invention describes a rapid, sensitive, and specific method for determining the presence and/or concentration of polyvalent and/or multivalent antigenic substances of the fungus P. herpotrichoides in a sample. The method also

employs a novel grinding apparatus for extraction of the antigen from plant material.

In the context of this disclosure, terms shall be defined as follows. "Fungal antigen" refers to any fungal substance or group of fungal substances which are capable of eliciting an immune response when injected into animals. Fungal antigens are discussed extensively by Longbottom and Austwick in Immunochemistry [ed. by Weir et al., Ch. 7 (1982)]. The term "immunogenic" refers to the intrinsic capacity of an antigen to elicit the production of antibodies when injected into an animal host. The term "polyvalent" refers to an antigen molecule having different immunogenic sites in response to which antibody molecules are produced. The term "multivalent" refers to an antigen molecule having multiple occurrences of a single immunogenic site. Among such antigenic substances may be mentioned polysaccharides, proteins, nucleic acids, lipids, and any aggregate or regularly ordered structure comprised of multiple or repeating units or combinations of such substances (e.g., glycoproteins, lipopolysaccharides, and lipoproteins). Among the specific fungal antigens which may be assayed by the process of the present invention are components of spores, hyphae, or cytosol including structural components, toxins, enzymes, elicitors, or other pathogenic factors whether they are secreted, extracted, crude, or purified. "Antibody" relates to a blood serum protein of the globulin fraction formed in response to antigen. "Polyclonal antibody" refers to any preparation containing heterogeneous populations of antibody molecules produced by genetically unique, clonally distinct antibody producing cells, whether derived from the serum of an animal or prepared artificially by mixing together

different monoclonal antibodies. "Monoclonal antibody" refers to homogeneous preparations of antibody molecules produced by a single population of cloned antibody producing cells. "Reaction system" refers to the assembled components of the immunoassay described herein. "High titer" refers to the capacity of an antiserum to be active at dilutions of 1: 50,000 or greater.

Figure 1 shows the components of the immunoassay assembled on a solid support (SS). The capture antibody (C.Ab) is a monospecific polyclonal rabbit-anti-P. herpotrichoides antiserum (RAP) Ig that has captured Pseudocercosporella antigen (P.Ag) previously extracted from plant material into a buffer. A cross-reactive labelled antibody (L.Ab) is also bound to the Pseudocercosporella antigen (P.Ag) and also serves as the attachment point for a reporter mechanism. The avidin-enzyme conjugate (A-E), biotin (B), and the enzyme specific substrate (S) are collectively known as the reporter mechanism. An avidin-enzyme conjugate (A-E) was attached to the biotin of the labelled antibody (L.Ab). A substrate (S) introduced into the reaction system and specific for the enzyme (E) of the avidin-enzyme conjugate (A-E) produced a colored product (CP) which is measured by means of a spectrophotometer. As discussed in greater detail herein, the order in which the components are assembled on the solid support (SS) depends on the conditions under which the assay will be used and the objectives of the assay.

Extraction of Fungal Antigens from Plant Tissue

As P. herpotrichoides invades plant tissue, antigens are produced by the fungus. These antigens must be extracted from fungal and plant matrices before they can be detected by the assay. A consistent extraction method is required if the assay is to be both sensitive and reproducible. Since the assay may be performed with both young and old plant tissue, as well as with dried straw, an extraction procedure capable of macerating fibrous material is required.

The present invention describes several ways of extracting fungal antigens from all types of plant materials. The two major components of the extraction system are the extraction solvent and the extraction apparatus. The solvent may be virtually any aqueous medium. Water alone suffices, however various additives may improve assay performance. The addition of pH buffering agents such as di- and mono-basic sodium phosphate or tris[hydroxymethyl]-aminomethane (Tris) help maintain neutral pH in the sample and yield more consistent results. The outer surfaces of plant parts are often quite hydrophobic and do not easily wet with water. The addition of a surfactant or wetting agent, such as polyoxyethylene-sorbatan monolaurate (Tween 20), octyl phenoxy polyethoxyethanol (Triton X-100), or sodium dodecyl sulfate aids in the extraction of fungal antigens. It is often desirable to limit microbial growth in the extracted plant material, particularly if the samples are to be stored for any length of time prior to assaying. Including a microbial growth inhibitor such as sodium azide, chloroacetamide or sodium ethylmercurithiosalicylate (thimerosal) is generally sufficient. Preferably, the extraction buffer

consists of a Tris buffered saline (TBS) or phosphate buffered saline solution (PBS) with azide or chloroacetamide added as a preservative and Tween 20 added as a wetting agent.

Extraction of fungal antigens from plant parts may be assisted by means of mechanical maceration of the plant and fungal biomass in the presence of extraction buffer. Maceration of soft, decomposed, or very young tissue may be accomplished by any number of techniques, including using Potter or Dounce type glass homogenizers, crushing between two hard surfaces, or repeated freezing and thawing. Older plants and dried straw require more rigorous techniques, such as grinding in a mortar and pestle with or without the assistance of a grinding aid such as sand or powdered glass. These techniques are very labor intensive and do not lend themselves to use outside of the laboratory or to the processing of large numbers of samples. The present invention describes a tissue grinding apparatus specifically designed to enhance the extraction of fungal antigens from all types of plant materials.

Grinding between rough surfaces such as sand paper or knurled plastic partially macerates some fibrous materials but the technique is tedious, inefficient and does not permit simultaneous introduction of the plant material into an extraction buffer. For this reason a grinding apparatus (10) illustrated in Figures 7 through 14 was designed having three parts: a grinding tube (12), a one- or two-piece plunger (24), and a lid (22). The grinding tube (12) is closed at one end by a circular base (14), the interior surface of which is a first

grinding surface (16) consisting of ridges (18) radiating from the center of the base (14). The base (14) also has a central peg (20) that protrudes from the base (14) within the grinding tube (12) and along the tube's longitudinal axis. The head (26) of the plunger (24) provides a second grinding surface (28) of radiating ridges (18) that fits into the grinding tube (12) to contact the first grinding surface (16). The plunger head (26) may be made as a separate unit from the plunger handle (32) in which case interlocking key (48) prevents the handle (32) and plunger head (26) from rotating independently. The second grinding surface (28) is interrupted at its center by a cavity (30) which fits over the grinding tube's peg (20) along the longitudinal axis. Thus positioned, the plunger (24) is free to rotate within the grinding tube (12) and extends out from the top of the grinding tube (12) in a handle (32) having features such as grips (34) or a depression (38) to assist either hand (34) or machine (38) rotation. The design of the tube (12) and plunger (24) is such that unground tissue sample is held between the grinding surfaces (16) and (28) in grinding zone (40), while macerated tissue is flushed away from the grinding zone (40) by the extraction buffer into the separation zone (42). This separation is achieved by providing a restricted clearance space (44) between the inner wall of the tube (12) and the grinding head (26). In the present embodiment, the restricted clearance space (44) is 0.007 of an inch. An approximate range for the restricted clearance space is 0.005 to 0.013 inches.

The design of the grinding surfaces is critical to the consistent performance of this invention. Most designs result in clogging of the grinding surface with plant material, thus greatly reducing

grinding efficiency. The present invention has grinding surfaces (16 and 28) which do not clog. The grinding surfaces (16 and 28) have ridges (18) which extend radially from the center of each grinding surface. The ridges (18) on the base (14) of the tube (12) and the plunger grinding head (26) are complimentary. The two surfaces interlock and disengage repeatedly when one or both of the two grinding surfaces are rotated about the tube's longitudinal axis. The constant engaging/disengaging action causes the grinding surfaces to abrade and crush the plant tissue at their interface. As described above, previously ground tissue is swept away from the grinding zone (40) by the extraction buffer decreasing the possibility of clogging. The peg (20) and cavity (30) in the center of the apparatus (10) excludes plant material from the center of the grinding surfaces (16 and 28) where grinding is less efficient due to reduced rotational motion and the diminished size of ridges (18). The peg (20) and cavity (30) mechanism is collectively known as a grind enhancing and alignment means (31). In the present embodiment, the space labelled (46) between the central peg (20) and the inner wall of the cavity (30) is 0.005 inches. An approximate range for this space is 0.003 to 0.010 inches. This alignment space (46) is less than the space (44) between the inner wall of the tube and the plunger head. Thus, the peg and cavity work together to form a grind enhancing and alignment means (31).

Figure 14 illustrates a preferred embodiment of the grinding device. The one piece tool/handle in this embodiment eliminates assembly of the tool and enhances reliability and cleanability. In this embodiment the grinding surfaces (16 and 28) are not

perpendicular to the longitudinal axis of the tube (12). Rather they are at an angle such that extraction buffer and fungal antigens from the macerated plant material flow by gravity to the bottom of the grinding zone (40) and then into the separation zone (42) through the restricted clearance space (44). Further, the angled grinding surface also permits shedding of extraction buffer as the tool is withdrawn from the cup, thus avoiding spillage and enhancing cleanability. The angle employed is not critical and can vary from 10° to 45° from the horizontal, preferably between 15° and 25°.

There are 3 or more ribs (49) spaced equally around the plunger (24). The ribs strengthen the plunger and provide a mixing action during grinding.

The plunger handle (32) in this embodiment has an expanded upper portion (47) that fits closely within the tube (12) such that the plunger can freely rotate. The clearance between the expanded upper portion (47) and the tube (12) can correspond to the clearance space (44). This feature helps stabilize the plunger during grinding and prevents materials from splashing out of the tube.

The expanded upper portion (47) can have a hole (48) for sample removal. If desired, the expanded upper portion can be fitted with a lid and serve as a reservoir for the extraction buffer.

The grinder (10) may be constructed out of metal, ceramic, glass, or plastic materials. Since the preferred use of the grinder is as an inexpensive, disposable apparatus, injection molded plastics such as polypropylene, polystyrene, polyethylene, or polycarbonate are preferred. The grinder may also be used to macerate other

materials such as seeds or soil in which case a harder material such as glass-filled plastic is required. The lid (22) in Figure 7 is used to maintain the cleanliness of the tube (12) and to protect the fungal antigen once extracted.

Fungal Antigens

Antigens derived from P. herpotrichoides may be isolated from the natural environment or cultured in vitro under optimal conditions to enhance the expression of immunogenic and, preferably, diagnostic antigens of the fungus while minimizing the amount of non-specific material in the antigenic extract obtained. Culture media may be liquid, solid, synthetic, defined, complex, enriched, or supplemented or combinations of these including tissues or substances derived from or comprised of cereal plants, preferably wheat. Other culture considerations include temperature, light, atmosphere, aeration, length of incubation, and other factors obvious to those skilled in the art.

Specific antigenic substances may be isolated from live or killed cells. Fungal cultures can be conveniently separated into solid and liquid fractions by either filtration or centrifugation. Culture filtrates or supernatants may contain metabolites or extracellular fungal antigens. Preparation of these molecules generally requires concentration and specific procedures such as dialysis or ultrafiltration to separate out media components. The solid fraction can be further processed by mechanical or physical disruption (e.g., drying and grinding, homogenizing, freezing and

thawing and sonicating), extraction with solvents or detergents, or digestion with enzymes, acids, alkali or other chemical means. The processed solid fraction can be further divided into cell wall and cytoplasmic (cytosol) fractions as a result of such disruption. Specific substances can be further purified by methods such as chromatography, electrophoresis, precipitation, dialysis, filtration, or combinations of these and other techniques obvious to those skilled in the art. Thus, the antigen may be a spore, hyphae, cytoplasmic fraction, toxin, enzyme, exoantigen, elicitor, polysaccharide, protein, nucleic acid, or any aggregate or regularly ordered structure composed of multiple repeating units or combinations of such structures or substances.

Antibodies to Fungal Antigens

Fungal antigens may be injected directly into animals capable of making antibodies to that substance. Alternatively, the antigens may require further preparation to enhance their immunogenicity prior to injection. Such procedures include mixing antigens with an appropriate adjuvant or conjugating antigens to a suitable carrier molecule. Factors affecting the immune response to injected antigens include the dose, route, timing, number of injections, genetic constitution of the animal (e.g., species and strain) and physical nature of the antigen (e.g., polysaccharide vs. protein). The choice of these factors for the immunization protocol depends upon the desired characteristics of the resulting antibodies. Applicants' goal was to design an immunization protocol to yield antibodies that

react with high specificity and sensitivity to biologically relevant fungal antigens. In the case of plant fungal diseases, relevant antigens may include extracellular substances such as exoantigens or enzymes, factors involved in the pathogenesis of the disease such as elicitors, or components of the invading hyphae.

The sensitivity of sandwich immunoassays is dependent upon the binding affinity of the antibody for the antigen and the overall avidity or strength by which the antigen is bound to the solid phase. Antigens may interact at multiple sites with antibody immobilized on the solid phase. Thus, the strength with which polyvalent or multivalent antigens are bound increases exponentially as a function of the number of valences involved in their attachment. Similarly, polyclonal antibodies immobilized on the solid phase may be expected to bind polyvalent antigens with greater avidity than monoclonal antibodies due to the inherent heterogeneity of the polyclonal reagent. Greater sensitivity can be achieved in forward sandwich immunoassays by employing monoclonal antibodies having affinities for their respective antigens of at least 10^8 M^{-1} and, preferably, of 10^9 M^{-1}. [U.S. Pat. No. 4,486,530] However, it is well documented in the scientific literature that antibodies to certain antigens (e.g., polysaccharides) only have affinities ranging from 10^4 M^{-1} to 10^6 M^{-1}. This is particularly evident in the highly inbred strains of mice used for the production of monoclonal antibodies. From these considerations it may be expected that preparations of polyclonal antibodies would provide more sensitive immunoassays to detect such antigens.

Specificity of an antibody for an antigen is determined primarily by their complementary fit and the strength of the binding between the two molecules. With respect to specificity of binding, a preparation of monoclonal antibody is homogeneous whereas a polyclonal preparation is heterogeneous. Preparations of polyclonal antibodies can be made more specific by selectively removing those antibody molecules that cross-react. This can be done by immunoabsorption of such preparations with antigens to which the reagent cross-reacts. The specificity of polyclonal reagents can also be improved by selectively purifying only those antibody molecules specific for the antigen of interest by means of immunoaffinity chromatography. This method can be time consuming, result in low yields and loss of high affinity antibodies from the population. In contrast, immunoabsorption techniques remove cross-reactive antibodies in a one step process, do not deplete high affinity antibodies, and can result in high yields. As shown in the Examples, Applicants used immunoabsorption techniques to create a monospecific antibody reagent by removing cross-reactive antibodies.

The choice of animal species or strains in which to produce antibodies is a critical factor of the immunization protocol and directly effects the final performance of any immunoassay. The prerequisite step to developing an immunoassay is the production of a high titer antiserum. Antibody production to a given antigen is determined genetically and varies between species and between strains within a species. Presently, monoclonal antibodies are derived almost exclusively from mice.

As stated previously, the current art argues for the use of monoclonal (i.e., mouse) antibodies over polyclonal antibodies to increase both specificity and sensitivity of fungal immunoassays. However, mice, especially the highly inbred strains used for the production of monoclonal antibodies, are highly restricted in their capacity to make antibodies to many antigens, including polysaccharides which are the primary components of the hyphal cell wall. It has been demonstrated that mice produce low titer, low affinity antisera when injected with preparations of fungal antigens. Thus, it may not be possible to isolate monoclonal antibodies with the desired sensitivity and specificity to detect important diagnostic fungal antigens. In contrast, rabbits of the type used routinely for the production of antibody reagents are outbred animals which frequently exhibit strong antibody responses to antigens which mice respond poorly to, including fungal antigens. While the current art champions the potential of monoclonal antibodies to increase the specificity and sensitivity of fungal immunoassays it has failed to realize the limitations of this technology imposed on it by the genetics of the mouse immune system.

Considering the short time requirements and low cost associated with development of polyclonal reagents as well as the sensitivity and specificity of the resulting assay, polyclonal antibody reagents offer many advantages over monoclonal reagents.

High Titer Polyclonal Capture Antibody Monospecific for Pseudocercosporella Antigen

The present invention uses an immunoabsorption technique to increase specificity by eliminating the

cross-reactivity of rabbit-anti-P. herpotrichoides (RAP) polyclonal antiserum to non-Pseudocercosporella antigens. As used herein, the term "immunoglobulin" refers to antibody and "monospecific" refers to preparations of Ig that have been rendered specific for Pseudocercosporella by immunoabsorption with cross-reacting fungi. There are a number of ways to prepare immunoabsorbed Ig. Available techniques include liquid phase immunoprecipitation with soluble fungal antigens, solid phase immunoabsorption with insoluble fungal components, and solid phase immunoabsorption with fungal antigen-solid support matrix conjugates. Each of these techniques involve treating the RAP Ig preparation with antigens prepared from cross-reacting fungi in order to specifically bind and remove cross-reactive antibodies. Fungi which may be used to immunoabsorb a RAP Ig reagent include Septoria tritici, S. nodorum, G. graminis, F. novali, F. culmorum, F. rosium, Rhizoctonia solani, Rhyncosporium ceacalis, P. anguioides, P. aestiva, as well as many other fungi which grow on cereal plants as pathogens or saprophites. The resulting monospecific Ig preparation will not react with fungal extracts to which it has been absorbed, yet retains reactivity towards P. herpotrichoides antigens.

Solid Support Matrix Immunoabsorbant

Covalently attaching an antigen, including its derivatives and analogs, to a solid support depends upon the particular molecular architecture of both types of molecules. Antigens contain functional groups such as amines, amides, carboxyls, sulfhydryls, hydroxyls, and/or aldehydes to which a

support matrix with its own appropriate functional groups could be attached directly or indirectly. The attachment chemistry may vary depending upon the functional groups involved and the desirability of including a spacer arm between the antigen and the solid support. The resulting matrix must retain the ability to react with antibody.

In the case of fungal antigen-agarose matrices, a number of coupling chemistries are available. When hydroxyl groups are present upon two adjacent carbon atoms within the structure of carbohydrate molecules, they can be treated with sodium periodate to form two aldehyde groups. These groups can attach directly to amino-functional agarose through a Schiff's base intermediate which can be reduced to a stable carbon-nitrogen bond. Such reduction can be carried out by conventional means such as either sodium borohydride or sodium cyanoborohydride. This sequence of reactions, commonly referred to as "periodate oxidation and borohydride reduction", results in an antigen column capable of binding antibody.

Alternatively, carbohydrate could be attached to agarose through a spacer arm, such as 1,6-diaminohexane, diaminodipropylamine, or other bifunctional crosslinking agents after periodate oxidation. The use of a spacer arm can enhance the immunochemical reactivity of the conjugate. A number of crosslinking agents, both homo- and hetero-bifunctional, have been described in the literature and are available commercially [Pierce Bio-Research Products Technical Bulletin, "Double-Agents", Bifunctional Crosslinking Reagents, Vol.3 Pierce Chemical Co., Rockford, IL, U.S.A., 1982]. These

crosslinking agents can be used in conjunction with many different linkage strategies, depending on which functional groups are available for conjugation on both the antigen and solid support.

Carbodiimide crosslinking agents, such as 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide, can also be employed to link antigens with carboxyl groups to amino groups on amino-activated agarose or conversely, to link the amino groups of the antigen to carboxyl groups on carboxyl-activated agarose.

Alternatively, support matrices may be purchased from commercial suppliers which have been pre-treated with crosslinking reagents. These "activated" supports will react directly with antigen functional groups when mixed in the appropriate coupling buffers. Examples of activated agarose support matrices and the functional groups to which they react are listed in Table 1.

TABLE 1

MATRIX IMMOBILIZED	LIGAND
Cyanogen Bromide-Activated Agarose	Amines, Proteins, Nucleic Acids, Polysaccharides
Epoxy-Activated - Agarose	Amines, Hydroxyls, Polysaccharides, Thiols
Nitrophenyl Chloroformate - Agarose	Amines, Proteins
Aminohexanoic Hydroxy Succinimide - Agarose	Amines, Proteins
Thiol - Agarose	Thiols, Alkyl & Aryl Halides, C=O, C=C, C=N
Carbonyldiimidazole - Agarose	Amines, Proteins
Carboxymethyl Cellulose Hydrazide and Polyacrylamide Hydrazide	Amines, Proteins, Polysaccharides

The chemical reactions involved in binding antigen molecules, or their derivatives or analogs, to the solid support are known to those skilled in the art. The important features of a particular antigen-support matrix are (1) that it react with an antibody specific for fungal antigen, and (2) that the matrix be stable under conditions of storage and use, i.e., that it not release antigen. While covalent bonds have been formed to ensure stability, bonding can involve other types of interactions which result in stable combinations of antigen and support, such as ionic or hydrophobic bonds. Such bonding methods are within the scope of this invention. Additionally, the molecular structure of the antigen derivative actually incorporated onto the solid support can vary depending upon the particular bonding reactions used and the optional use of a spacer arm. Such molecular structural variations to achieve bonding are not critical to practice the invention. Thus, "matrix" means any antigen molecule attached, directly or indirectly, to a solid support.

Pseudocercosporella Immunoassay

The assay to detect P. herpotrichoides antigen in infected plant tissue may be configured in numerous formats. All formats require immobilization of antigen or capture antibody onto a solid support. The solid support matrices may be microtiter plates, tubes, beads, magnetic particles, membranes and other well known supports.

In the case of immobilized antigen, the plant extract is adsorbed directly to the solid support and then reacted with a labelled antibody specific for fungal antigen. Since both plant material and fungal

antigen adsorb to the support, the fungal antigen density on the solid support is limited by the presence of non-specific plant material. This usually results in low assay sensitivity. It is important to detect the presence of P. herpotrichoides in wheat stems before the expression of symptoms. Therefore, it is desirable to have an assay with high sensitivity. The use of immobilized capture antibody in a forward-sandwich assay as schematically shown in Figure 1 is understood to be more sensitive than reverse or simultaneous sandwich assays. The capture antibody (C.Ab) is adsorbed to the solid support (SS) and then reacted with plant extract. The capture antibody (C.Ab) serves to selectively immobilize the fungal antigen (P.Ag) from the plant extract and results in a high specific antigen density on the solid support. The bound fungal antigen (P.Ag) is then reacted with a labelled second antibody (L.Ab).

Several grades of immunoreagent may be used to perform the assay. Generally, purified Ig reagents improve assay performance. Assay specificity is achieved by the use of immunoabsorbed reagents. The capture antibody, second antibody, or both, may be immunoabsorbed. Using the capture antibody as the only immunoabsorbed reagent may result in slightly improved assay sensitivity over other configurations.

Reporter Mechanism

The choice of reporter mechanism used to indicate the presence of bound antigen includes radioisotopes, fluorescent chromophores, chromophoric particles and enzymes. Enzymes are preferred due to the availability of sensitive chromogenic substrates

and the availability of simple instrumentation to quantitate results. The use of horseradish peroxidase may be limited due to the presence of endogenous peroxidase activity in plant and fungal extracts which can result in high non-specific color development. Alkaline phosphatase is the preferred label and its use results in low nonspecific color development and excellent assay sensitivity. For use in microtiter plate assays, the applicants used the soluble yellow color signal produced by the enzyme substrate p-nitrophenyl phosphate and measured the signal as described in Examples 1g, 1h, 1i and 2. With other assay formats, 5-bromo-4-chloro-3-indolyl phosphate, p-toluidine salt may be used in conjuction with a tetrazolium salt, such as nitroblue tetrazolium chloride, to yield an insoluble formazan dye precipitate. In either case, the color signal is related to the presence or concentration of fungal antigen initially present in the aqueous medium.

The reporter mechanism may be prepared using several techniques. The first technique requires the use of chemical conjugation methods similar to those described in the "Antigen Support Matrix Immunoabsorbant" section above. The result is an immunoglobulin molecule covalently coupled to one or more label molecules. Alternatively, immunoglobulin and the label may be coupled via non-covalent techniques, such as with the use of a streptavidin-biotin system or the avidin-biotin system as taught in U.S. Patent No. 4,228,237.

Field Testing Kit

Applicants' kit format for their invention permits convenient, fast testing in the field of

cereal crops suspected of being infected with the Pseudocercosporella fungus. The kit will include the grinding device and the preparations of the various assay components described herein.

EXAMPLES

Unless specified otherwise, in all statements of assay conditions and the preparation of reagents the following are meant: temperature is in °C; concentrations referred to as percentages are by weight/volume; and abbreviations utilized are: ml (milliliter), ul (microliter), M (molar), mM (millimolar), g (gravity), MW (molecular weight), rpm (revolutions per minute), nm (nanometer), um (micrometer), h (hour), min (minute).

EXAMPLE 1a

Preparation of Fungal Extracts

Separate culture extracts were prepared from five species of pathogenic wheat fungi. The extracts served as both individual antigen sources for immunoassay testing and as a source of antigen for the immunoabsorption procedure described in Example 1f.

Culture flasks containing 100 ml of sterile Potato Dextrose Broth (Difco, Detroit, MI) were inoculated with the spores and hyphae from one of the following fungi: P. herpotrichoides, S. tritici, F. roseum, R. solani, or G. graminis. Cultures were shaken at 100 rpm for one to two weeks at 23°. Following the incubation period, hyphae and culture supernatants were separated by centrifugation at 3000xg for 10 min and stored frozen at -20°.

Fungal antigens were then prepared by sodium cholate extraction of the spent culture media and

macerated hyphae. Culture medium was added to hyphal pellets to result in a hyphal suspension of approximately 1 ml of media per 1 ml of packed hyphae. The hyphal suspensions were adjusted to 0.1% sodium cholate by the addition of a 1% stock solution in PBS (20 mM sodium phosphate, 140 mM sodium chloride) containing 0.05% sodium azide (PBS/azide). The hyphae were thoroughly homogenized in Dounce-type tissue grinders. Homogenates were centrifuged at 3000xg for 10 min at 4° and the resulting cell pellets were discarded. The supernatant fractions were combined with the remainder of the spent culture media and the mixtures were dialyzed exhaustively against two changes of saline (140 mM sodium chloride) followed by a final buffer change into coupling buffer (100 mM sodium bicarbonate, 500 mM sodium chloride, pH 8.5) using dialysis membranes with an 8,000 MW cutoff. The dialyzed extracts were concentrated to approximately 10 ml by ultra-filtration in stirred-cells (Amicon, Bedford, MA) using 10,000 MW cut-off membrane filters.

EXAMPLE 1b

Extraction of Fungal Antigens from Plant Tissue

Both greenhouse- and field-grown wheat plants were tested separately in the assay. Antigen sources included fresh, frozen and dried plants. Plants were prepared for extraction by cutting one to two inch stem segments measured up the plant from the basal culm to just above the coleoptile. Stems were extracted in PBS containing 0.05% Tween 20 (PBS/Tween) using a mortar and pestle. The resulting plant extracts were centrifuged at 3000xg for 1 min to remove large debris and stored at 4° until

assayed. The extract derived from the ten stem segment in 1 ml of extraction buffer is defined as "undilute". In the present example, one field-collected stem segment extracted into 5 ml of buffer is defined as a 1:50 starting dilution and one green house collected stem segment extracted into 10 ml of buffer is defined as a 1:100 starting dilution.

EXAMPLE 1c

Preparation of Immunogens and Rabbit Immunization Protocol

A first immunogen was prepared by culturing four isolates of P. herpotrichoides, including wheat, rye and Pacific Northwest strains in Potato Dextrose Broth (Difco). The cultures were homogenized and fungal fragments counted using a microscope and hemacytometer. All four strains were diluted in PBS and pooled to yield a mixture containing approximately equal numbers of fragments of each of the four strains. The pooled mixture (4×10^5 fungal fragments per ml) was emulsified with an equal volume of either complete Freund's adjuvant (CFA) or incomplete Freund's adjuvant (IFA) prior to injection into the animals. Three New Zealand white rabbits were given two 1 ml injections of the fungal-adjuvant emulsion subcutaneously (SC) at multiple sites on days 0 (CFA) and 24 (IFA).

A second antigen was prepared by grinding into powder the lyophilized hyphae of P. herpotrichoides (a wheat strain) grown in mineral dextrose yeast (MDY) broth. The powdered hyphae (1 gram) was resuspended in 10 ml PBS and centrifuged to pellet insoluble material. The clarified supernatant (optical density at 280 nm = 4.9) was sterilized by

filtration through a 0.45 um membrane (Gelman, Ann Arbor, MI) and used to immunize previously injected New Zealand white rabbits. Each rabbit was injected with 0.5 ml antigen B intravenously (IV) and 0.5 ml emulsified with 0.5 ml IFA intramuscularly (IM) on day 81. Antiserum was harvested from rabbits bled on days 0, 39, 49, 88, 91 and 95. A third immunogen (C) was prepared from a wheat strain fungal extract (Example 1a). The concentrated extract was centrifuged at 7800xg for 20 min and the supernatant sterile filtered. The sterile supernatant was emulsified with an equal volume of IFA and injected on days 140 and 168 (0.5 ml IM and 1.0 ml SC). Antisera were harvested from rabbits bled on days 154, 182 and 187.

EXAMPLE 1d

Purification of Rabbit-anti-*P. herpotrichoides* Immunoglobulin (RAP Ig)

Purified RAP Ig was prepared from pooled antisera harvested from two of the rabbits bled on days 182 and 187. Fifty-five ml of antisera were filter sterilized, mixed with an equal volume of saturated ammonium sulfate and incubated 1 h at 4°. The precipitated Ig was harvested by centrifugation, dissolved in a small volume of saline and dialyzed in 14,000 MW cutoff dialysis tubing 48 h in two changes of PBS at 4° (2 liters each change). The dialyzed Ig solution was centrifuged at 3000xg for 20 min to remove precipitated material. A Protein A Sepharose 4B slurry (32 ml, Pharmacia, Piscataway, NJ) was poured into a column (2.5 x 20 cm) and equilibrated with 300 ml 3 M NaCl, 1.0 M Glycine, pH 8.8 (U.S. Patent No. 4,704,366). The clarified Ig solution was

mixed with an equal volume of equilibration buffer and allowed to pass through the column at a rate determined by gravity (approx. 1 ml/min). The column was washed with equilibration buffer until the absorbance at 280 nm reached the baseline. Antibody was eluted from the column with 53 ml of 0.1 M sodium citrate, pH 3.0 and dialyzed overnight at 4° in two changes of PBS (4 liters each change).

The concentration of purified Ig was determined using an extinction coefficient E(1%, 280 nm)=14.3.

EXAMPLE 1e

Preparation of Biotinylated Rabbit-anti-P. herpotrichoides Immunoglobulin Conjugate

Purified RAP Ig (100 mg, 5 mg/ml PBS) was diluted with an equal volume of 0.1 M bicarbonate buffer pH 8.1. The diluted Ig was dialyzed with 14,000 MW cutoff dialysis tubing for 24 h at 4° in two changes of 6 liters 0.1 M bicarbonate buffer. N-Hydroxy-succinimidobiotin (Pierce, Rockford, IL) was dissolved in dimethyl sulfoxide (5 mg/ml) and 4.2 ml reacted with the dialyzed Ig (40 ml) for 4 h at room temperature. The resulting conjugate solution was dialyzed against two changes of PBS (6 liters each change) for 96 h at 4°. The biotin-Ig conjugate was prepared for storage at 4° by addition of bovine serum albumin (1% final concentration), glycerol (10% v/v), sodium azide (0.1%) and sterile filtration.

EXAMPLE 1f

Immunoabsorption of Cross-reactive Rabbit-Anti-P. herpotrichoides Immunoglobulin (RAP Ig)

The following protocol illustrates that purified RAP Ig cross-reacts with antigens prepared

from numerous other plant pathogenic fungi when tested using a forward-sandwich enzyme immunoassay (Figure 3).

The cross-reactive Ig was removed by a solid phase immunoabsorption technique to yield monospecific RAP Ig. Fungal extracts from Example 1a of S. tritici, F. roseum, R. cerealis, and G. graminis were coupled separately to cyanogen bromide-activated Sepharose 4B (Pharmacia AB, Laboratory Separation Division, Uppsala, Sweden). For each preparation, one gram of dried gel was suspended in 33 ml of 1 mM hydrochloric acid (HCl) and transferred to a Buchner funnel. The gel was washed continuously with a total of 300 ml of HCl over 15 minutes. After the acid wash, the gel was rapidly filtered down to a moist paste, transferred to a polypropylene centrifuge tube, resuspended into 1 gel bed volume of coupling buffer, and centrifuged at 500 x g for two minutes. The gel pellet was saved and immediately combined with approximately 10 ml of fungal extract. The antigens were allowed to react with the activated gel matrix for 2.5 h at room temperature for Septoria, Fusarium, and Gaeumannomyces or overnight at 4° for Rhizoctonia, with gentle mixing under both conditions. Following incubation, the gel was washed twice with 1 ml of coupling buffer. Remaining reactive groups on the Septoria, Fusarium, and Gaeumannomyces gels were blocked using 1 M ethanolamine, pH 8.0, for 2 h at room temperature. Reactive groups on the Rhizoctonia gel were blocked using 10 mM Tris, pH 7.4, 140 mM sodium chloride, 0.05% sodium azide (TBS/azide) incubated overnight at 4°. The fungal antigen-Sepharose 4B immunoabsorbants were packed

into 1.0 x 10 cm chromatography columns and sequentially washed with 0.1 M sodium acetate, 1 M sodium chloride, pH 4.0, and 0.1 M sodium carbonate, 1 M sodium chloride, pH 9.0, and 3 M sodium thiocyanate in PBS followed by PBS/azide prior to use. Final column bed volumes were 3-5 ml.

Purified RAP Ig at 5.2 mg/ml in PBS was sequentially passed through the G. graminis, S. tritici, F. roseum, and R. solani immunoabsorbant columns. The Ig preparation was allowed to pass through the columns at approximately 1 ml/min. The columns were washed with PBS/azide until the absorbance of the column effluents at 280 nm returned to zero. Each pass through an affinity column resulted in a 2-3 fold dilution of the RAP Ig which was concentrated back down to the approximate starting volume in a stirred-cell using a 100,000 MW cut-off membrane filter.

Figure 4 is a bar graph showing the results of absorbed monospecific RAP Ig used as capture antibody in a forward sandwich enzyme immunoassay and demonstrating cross-reactivity to fungal extracts of Septoria, Fusarium, and Gaeumannomyces antigens.

EXAMPLE 1g

Pseudocercosporella Immunoassay

The Pseudocercosporella Immunoassay on plant tissue suspected of being infected with P. herpotrichoides was performed. The format consisted of a 96 well microtiter plate. The general assay protocol, consisted of 50 ul reagent additions per well, 1 h reagent incubations at 25° followed by four washes with 10 mM Tris, pH 7.4, 140 mM sodium chloride, 0.05% sodium azide, 0.05% Tween 20 (TBS/Tween). RAP Ig (cross-reactive or monospecific)

was diluted to approximately 10 ug/ml in 10 mM bicarbonate coating buffer at pH 9.6 and added to microtiter plate wells. Samples were diluted in TBS/Tween and incubated with antibody coated wells. After the plates were incubated and washed, biotin-conjugated RAP Ig diluted to approximately 10 ug/ml in TBS/Tween was applied to the wells. Final reagent additions consisted of avidin-alkaline phosphatase conjugate (Organon Technica, Malvern, PA) diluted 1:250 into TBS/Tween followed by phophatase substrate solution (1 mg/ml p-nitrophenyl phosphate, 2.0 mM magnesium chloride, 0.2 mM zinc acetate in 1.0 M diethanolamine buffer, pH 9.8). Color development in the wells was measured spectrophotometrically at 405 nm.

EXAMPLE 1h

Applications of the Pseudocercosporella Immunoassay

Figures 2a and 2b illustrate the sensitivity of the Pseudocercosporella Immunoassay for detecting the presence of P. herpotrichoides infection before the appearance of symptoms in both greenhouse- (Figure 2a) and field- (Figure 2b) infected wheat stems. Samples were prepared according to Example 1b.

Figures 3 and 4 illustrate the specificity of the Pseudocercosporella Immunoassay for P. herpotrichoides when monospecific RAP Ig is used to coat the surface of microtiter plates. Wells were coated with unabsorbed (Figure 3) or monospecific (Figure 4) RAP Ig and reacted with fungal extracts described in Example 1a of S. tritici, F. roseum, P. herpotrichoides, and G. graminis diluted 1:100 in TBS/Tween.

Figure 5 shows that the assay is sensitive enough to detect picogram quantities of P. herpotrichoides antigen. The concentration of P. herpotrichoides antigen was determined by dialyzing a wheat strain fungal extract, against PBS as in Example 1b, evaporating a 0.5 ml sample to dryness at 37°, determining the weight of the dried residue and calculating the weight of the P. herpotrichoides antigen by subtracting out the weight of the salt.

EXAMPLE 1i

Microtiter Plate Pseudocercosporella Immunoassay

The general assay protocol, following procedures described in Example 1g, consisted of 100 µl reagent additions per well, 30 min reagent incubations at 25° followed by three washes with 10 mM Tris, pH 7.4, 140 mM sodium chloride, 0.04% chloroacetamide, and 0.05% Tween 20 (TBSC/Tween). Monospecific RAP Ig was diluted to approximately 10 µg/ml in 0.1 M bicarbonate coating buffer at pH 9.6 and added to microtiter plate wells. Samples were diluted in TBSC/Tween and incubated with antibody coated wells. After the plates were incubated and washed, biotin-conjugated RAP Ig diluted to approximately 5 µg/ml in TBSC/Tween was applied to the wells. Final reagent additions consisted of streptavidin-alkaline phosphatase conjugate diluted to approximately 1 µg/ml in TBSC/Tween followed by p-nitrophenyl phosphate substrate solution (Kirkegaard and Perry Laboratories, Inc.). Color development in the wells was measured spectrophotometrically at 405 nm.

EXAMPLE 1j

Membrane Format Pseudocercosporella Immunoassay

Membrane based immunoassays were performed in a Fast-Chek™ device (E-Y Labs, Inc., San Mateo, CA). The device consists of a porous nitrocellulose membrane backed with several pieces of absorbant paper encased within a plastic body. The surface of the nitrocellulose membrane serves as the solid support for immobilizing the capture antibody. Reagents applied to the surface of the solid support wick through the pores of the membrane into the absorbant paper backing. Reagents and buffers were identical to those described in Example 1i except where noted. One µl of a 0.94 mg/ml solution of monospecific RAP Ig was applied to the nitrocellulose membrane. After drying the membrane for 5 min, 100 µl of blocking solution (Buffer C, E-Y Labs, Inc.) was applied and allowed to wick completely through the membrane. 100 µl of each additional reagent and wash buffer (as described in Example 1i) were applied sequentially and allowed to completely wick through the membrane before the next reagent was applied. The alkaline phosphatase substrate 5-bromo-4-chloro-3-indolyl phosphate, p-toluidine salt was used in conjunction with nitroblue tetrazolium chloride (BCIP/NBT Phosphatase Substrate, Kirkegaard and Perry Labs, Inc.) to yield an insoluble blue precipitate on the membrane.

EXAMPLE 2

Application of Tissue Grinder to Pseudocercosporella Immunoassay for Extraction of Fungal Antigens from Plant Tissue

Fungal antigens were extracted from plant tissue as described in Example 1b except that field

collected plant material was macerated into 5 ml of extraction buffer using the tissue grinding apparatus described in Figures 7 through 13.

The assay was performed in accordance with the Examples 1a, and 1c through 1h.

Color development in the microtiter plate wells was measured spectrophotometrically at 410 nm as shown in Figure 6.

It will be apparent that the instant specification and examples are set forth by way of illustration and not limitation, and that various modifications and changes may be made without departing from the spirit and scope of the present invention.

CLAIMS

What is claimed is:

1. A grinding device, comprising:

a cylindrical tube, one end of the tube being closed with a circular base, the base having on its interior face a plurality of radiating ridges constituting a first grinding surface; and

a plunger having a first end and a second end, the first end being cylindrical and having radiating ridges complementary to the ridges of the first grinding surface constituting a second grinding surface, the first end of the plunger slidably insertable into the cylindrical tube and axially rotatable therein, the second end of the plunger having a means for rotating the plunger within the cylindrical tube to grind material positioned between the first and second grinding surfaces, the space between the inner surface of the tube and the first end of the plunger such that ground material escapes from the first and second grinding surfaces along the inner surface of the tube but unground material does not escape from the first and second grinding surfaces.

2. The grinding device of Claim 1 having in addition a grind enhancing and alignment means, the grind enhancing and alignment means comprising:

a peg protruding concentrically along the tube's longitudinal axis from the interior face of the base; and

a cavity recessed concentrically along the plunger's longitudinal axis from the first end of the plunger, the cavity having dimensions such that

the plunger rotatably fits over the peg when the first grinding surface contacts the second grinding surface.

3. The grind enhancing and alignment means of Claim 2, wherein the space between the peg and the inner wall of the cavity is less than the space between the inner wall of the tube and the outer surface of the plunger head.

4. The grinding device of Claim 1 wherein the circular base is perpendicular to the longitudinal axis of the tube.

5. The grinding device of Claim 1 wherein the first and second grinding surfaces are at an angle of 15°-25° from the horizontal.

6. The grinding device of Claim 1 wherein the second end of the plunger has a close, rotating fit within the tube.

7. A method for assaying the fungus of the genus Pseudocercosporella, comprising:

A. extracting fungal antigens from plant tissue into an aqueous medium;

B. forming a reaction system on a solid support coated with a high titer polyclonal capture antibody that is reactive with the fungus Pseudocercosporella, and contacting said surface with :

1. the aqueous medium containing the fungal antigen;

2. then adding a polyclonal antibody that reacts with the antigen;
3. then adding a reporter mechanism;

C. Measuring the signal from the reporter mechanism; and

D. Relating the signal to the presence or concentration of fungal antigen intitially present in the aqueous medium.

8. The method of Claim 7 wherein the plant tissue is obtained from plants susceptible to infection by a member of the genus Pseudocercosporella.

9. The method of Claim 7 wherein the fungal antigen is selected from the group consisting of a Pseudocercosporella herpotrichoides spore, hypha, cytoplasmic fraction, toxin, enzyme, exoantigen, elicitor, polysaccharide, protein, nucleic acid, or any aggregate or regularly ordered structure comprised of multiple or repeating units or combinations of such structures or substances.

10. The method of Claim 7 wherein the fungal antigen is a Pseudocercosporella polysaccharide.

11. The method of Claim 7 wherein the aqueous medium preferably comprises a phosphate buffered saline solution, azide, and polyoxyethylenesorbatan monolaurate.

12. The method of Claim 7 wherein the solid support is selected from the group consisting of microtiter plates, tubes, beads, magnetic particles, and membranes.

13. The method of Claim 7 wherein the solid support is a polystyrene microtiter plate.

14. The method of Claim 7 wherein the high titer polyclonal capture antibody reagent is monospecific for the genus Pseudocercosporella.

15. The method of Claim 7 wherein the reactive polyclonal antibody is labelled with a substance selected selected from the group consisting of enzymes, radioisotopes, chromophoric particles and fluorescent chromophores.

16. The method of Claim 7 wherein the reactive polyclonal antibody is labelled with a biotin-avidin-enzyme unit.

17. The method of Claim 7 wherein the reactive polyclonal antibody is labelled with alkaline phosphatase.

18. The method of Claim 7 wherein the reporter mechanism comprises a biotin-avidin-enzyme conjugate coupled to the reactive polyclonal antibody and a substrate specific to the enzyme, the reporter mechanism indicating a color signal in the presence of Pseudocercosporella antigen.

19. The method of Claim 18 wherein the enzyme is alkaline phosphatase.

20. The method of Claim 18 wherein the enzyme substrate is p-nitrophenyl phosphate.

21. A method for assaying the fungus Pseudocercosporella herpotrichoides, comprising:

A. extracting a Pseudocercosporella antigen from plant tissue into a solution of Tris-buffered saline, chloroacetamide, and polyoxyethylene-sorbitan monolaurate;

B. forming a reaction system on polystyrene microtiter plate coated with a high-titer polyclonal capture antibody monospecific for the Pseudocercosporella antigen and contacting said microtiter plate with:

1. the solution containing the fungal antigen;
2. then adding a polyclonal antibody-biotin conjugate that reacts to the Pseudocercosporella antigen;
3. then adding a reporter mechanism including a streptavidin-alkaline phosphatase conjugate and p-nitrophenyl phosphate,

in proportions such that a signal from the reporter mechanism is related to the presence of or concentration of Pseudocercosporella antigen initially present in the aqueous medium;

C. Measuring the signal from the reporter mechanism; and

D. Relating the signal to the presence or concentration of Pseudocercosporella antigen intitially present in the aqueous medium.

22. A method for assaying the fungus Pseudocercosporella herpotrichoides, comprising:

A. extracting a Pseudocercosporella antigen from plant tissue into a solution of Tris-buffered saline, chloroacetamide, and polyoxyethylene-sorbitan monolaurate;

B. forming a reaction system on a membrane coated with a high-titer polyclonal capture antibody monospecific for the Pseudocercosporella antigen and contacting said membrane with:

1. the solution containing the fungal antigen;
2. then adding a polyclonal antibody-biotin conjugate that reacts to the Pseudocercosporella antigen;
3. then adding a reporter mechanism including a streptavidin-alkaline phosphatase conjugate and 5-bromo-4-chloro-3-indolyl phosphate substrate,

in proportions such that a signal from the reporter mechanism is related to the presence of or concentration of Pseudocercosporella antigen initially present in the aqueous medium;

C. Measuring the signal from the reporter mechanism; and

D. Relating the signal to the presence or concentration of Pseudocercosporella antigen initially present in the aqueous medium.

23. The method of Claim 7 wherein the aqueous medium preferably comprises a Tris-buffered saline solution, chloroacetamide and polyoxyethylenesorbitan monolaurate.

24. The method of Claim 7 wherein the solid support is a membrane.

25. The method of Claim 7 wherein the reactive polyclonal antibody is labelled with a biotin-streptavidin-enzyme unit.

26. The method of Claim 7 wherein the reporter mechanism comprises a biotin-streptavidin-enzyme conjugate coupled to a reactive polyclonal antibody and a substrate specific to the enzyme, the reporter mechanism indicating a color signal in the presence of Pseudocercosporella antigen.

27. The method of Claim 26 wherein the enzyme is alkaline phosphatase.

28. The method of Claim 26 wherein the enzyme substrate is 5-bromo-4-chloro-3-indolyl phosphate.

29. A kit to test for the presence of Pseudocercosporella antigen in cereal crop, comprising:

a tissue grinder for the extraction of Pseudocercosporella antigen from plant tissue;

antigen extraction buffer in which to place the antigen;

a solid support for the attachment of an immunoassay;

a preparation of labelled antibody which includes an enzyme and an antibody reactive with the Pseudocercosporella antigens;

a preparation of antibody monospecific for Pseudocercosporella antigens; and

a preparation of enzyme substrate which is specific for the enzyme of the labelled antibody preparation and indicates this specificity by a color change.

30. A kit of Claim 29 wherein:

the antigen extraction buffer is Tris-buffered saline, chloroacetamide, and polyoxyethylene-sorbitan monolaurate;

the solid support is a polystyrene microtiter plate;

the labelled antibody is a system comprising a polyclonal antibody-biotin conjugate that reacts to the Pseudocercosporella antigen and a streptavidin-alkaline phosphatase conjugate reporter mechanism; and

the enzyme substrate is p-nitrophenyl phosphate.

31. A kit of Claim 29 wherein:

the antigen extraction buffer is Tris-buffered saline, chloroacetamide, and polyoxyethylene-sorbitan monolaurate;

the solid support is a membrane;

the labelled antibody is a system comprising a polyclonal antibody-biotin conjugate that reacts to the Pseudocercosporella antigen and a streptavidin-alkaline phosphatase conjugate reporter mechanism; and

the enzyme substrate is 5-bromo-4-chloro-3-indolyl phosphate.

FIG. 1

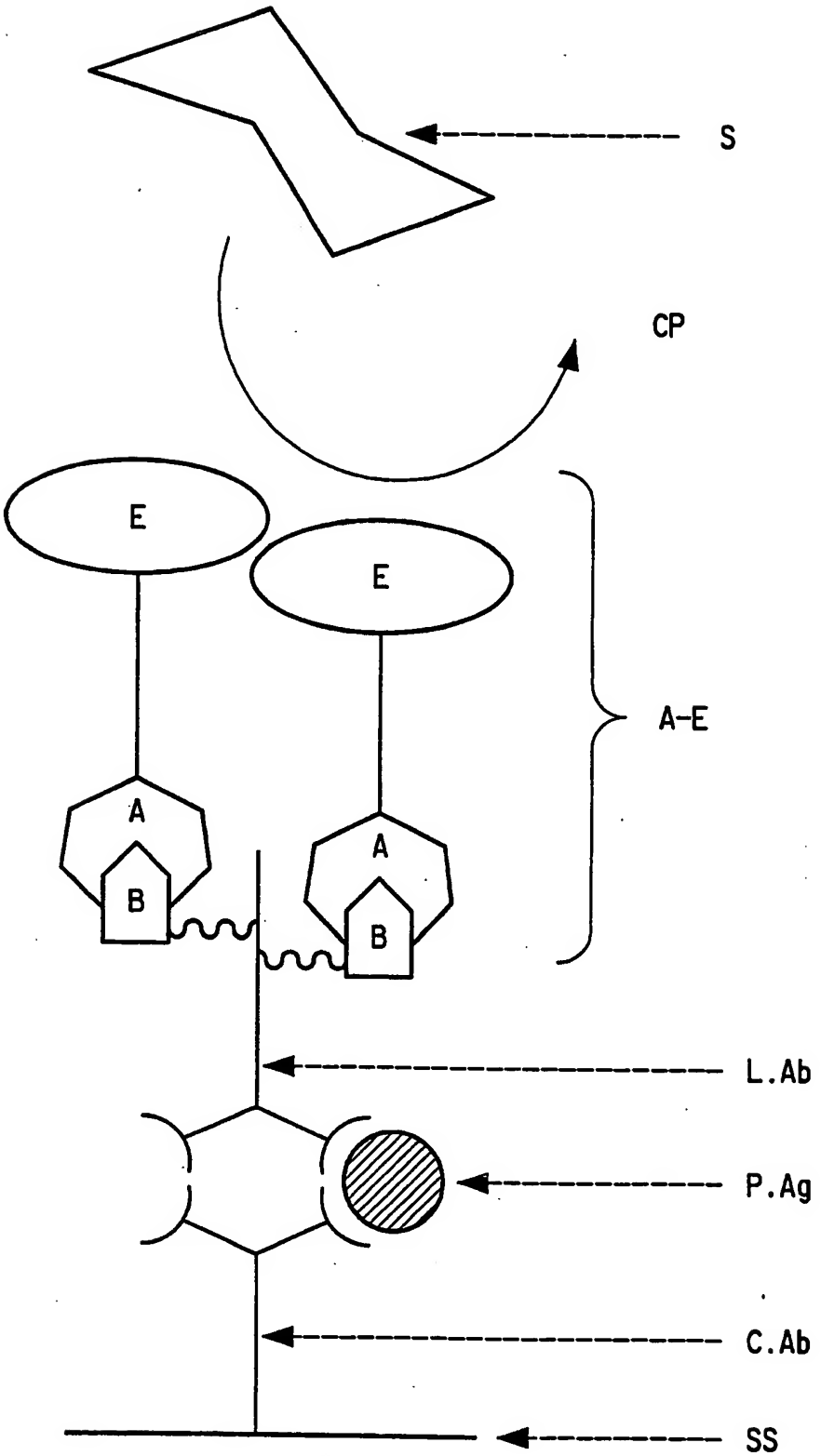


FIG.2A

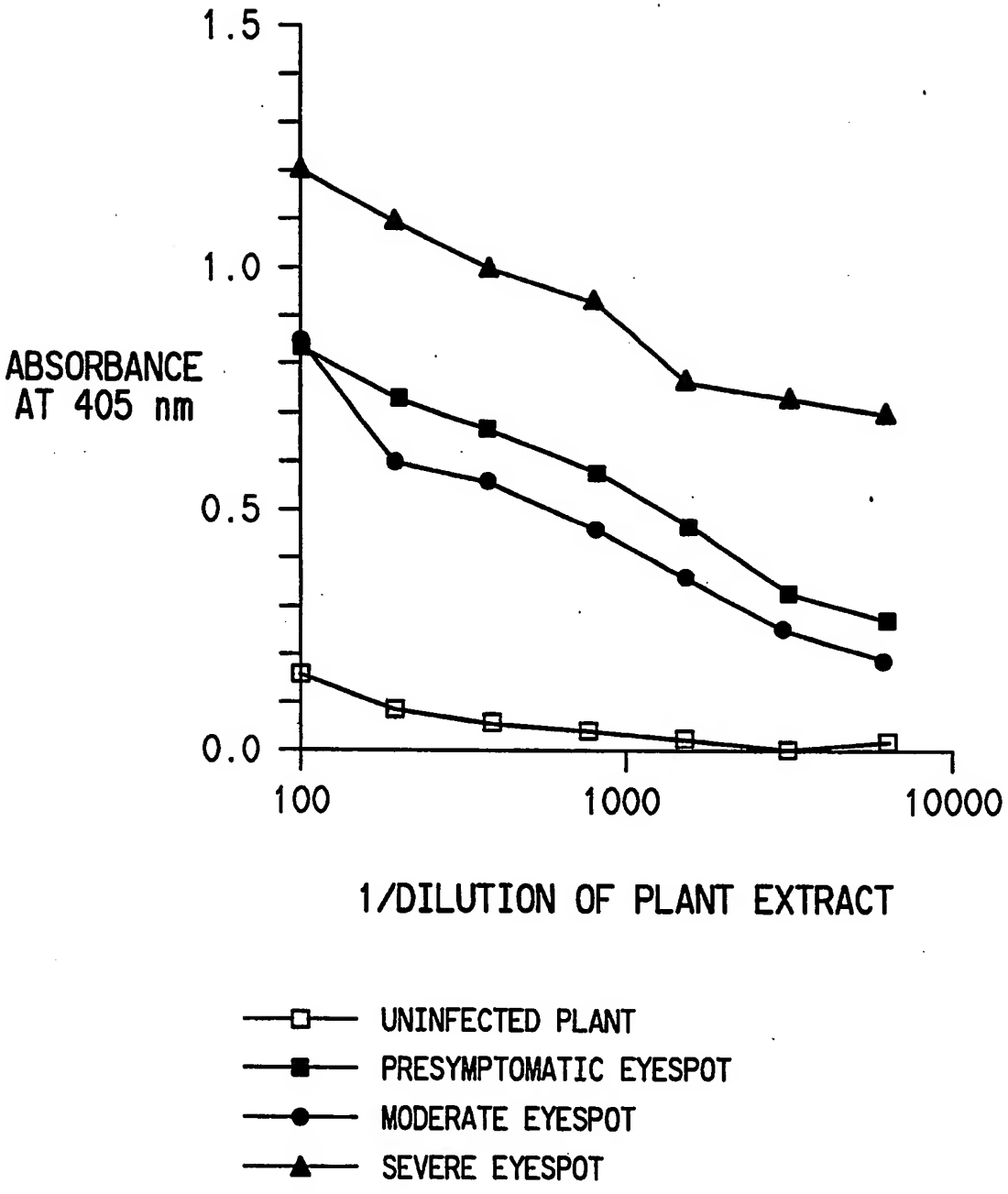

1.5
1.0
0.5
0.0
ABSORBANCE AT 405 nm
100
1000
10000
1/DILUTION OF PLANT EXTRACT
UNINFECTED PLANT
PRESYMPTOMATIC EYESPOT
MODERATE EYESPOT
SEVERE EYESPOT

FIG.2B

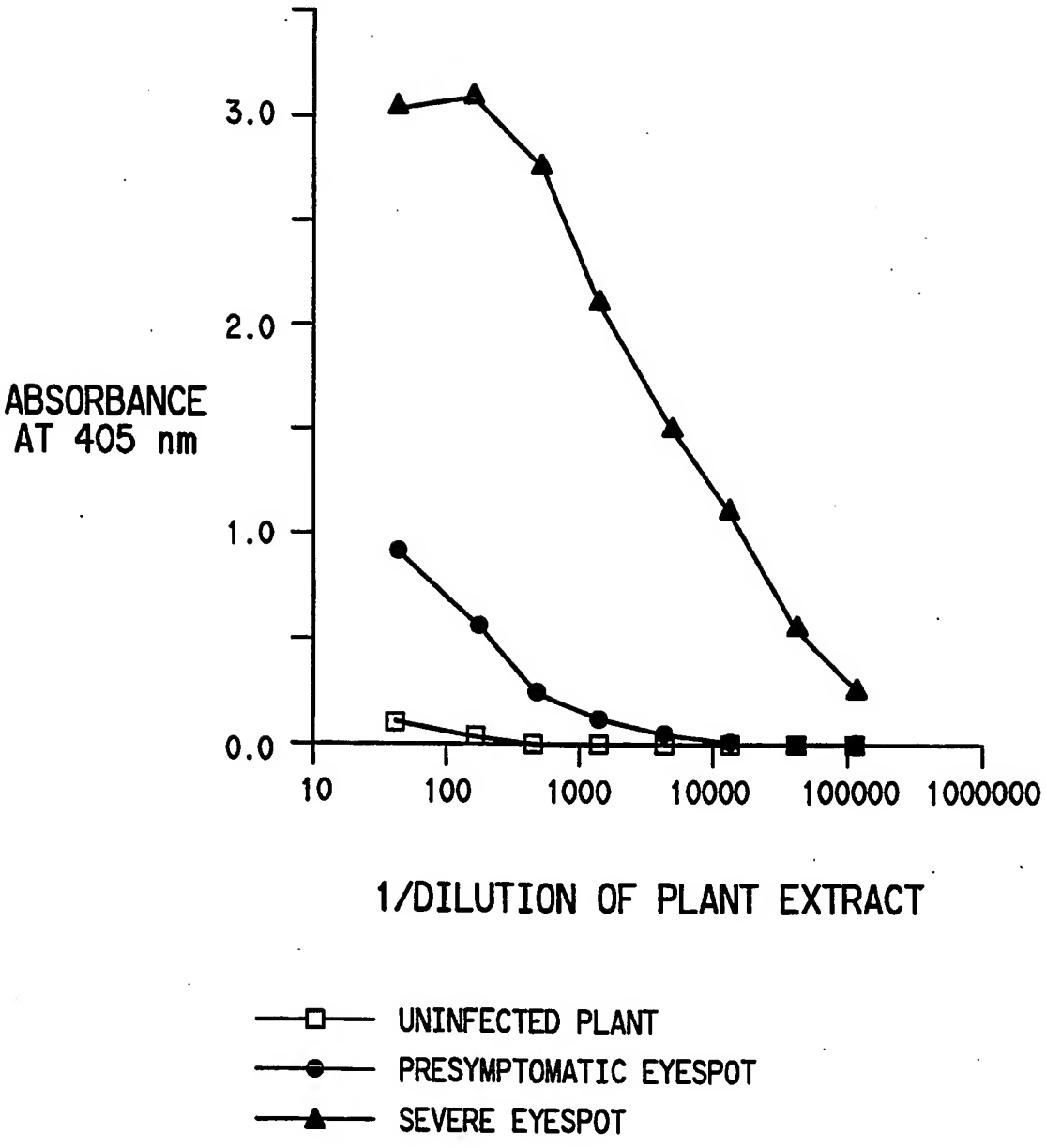

ABSORBANCE AT 405 nm
3.0
2.0
1.0
0.0
10
100
1000
10000
100000
1000000
1/DILUTION OF PLANT EXTRACT
UNINFECTED PLANT
PRESYMPTOMATIC EYESPOT
SEVERE EYESPOT

FIG.3

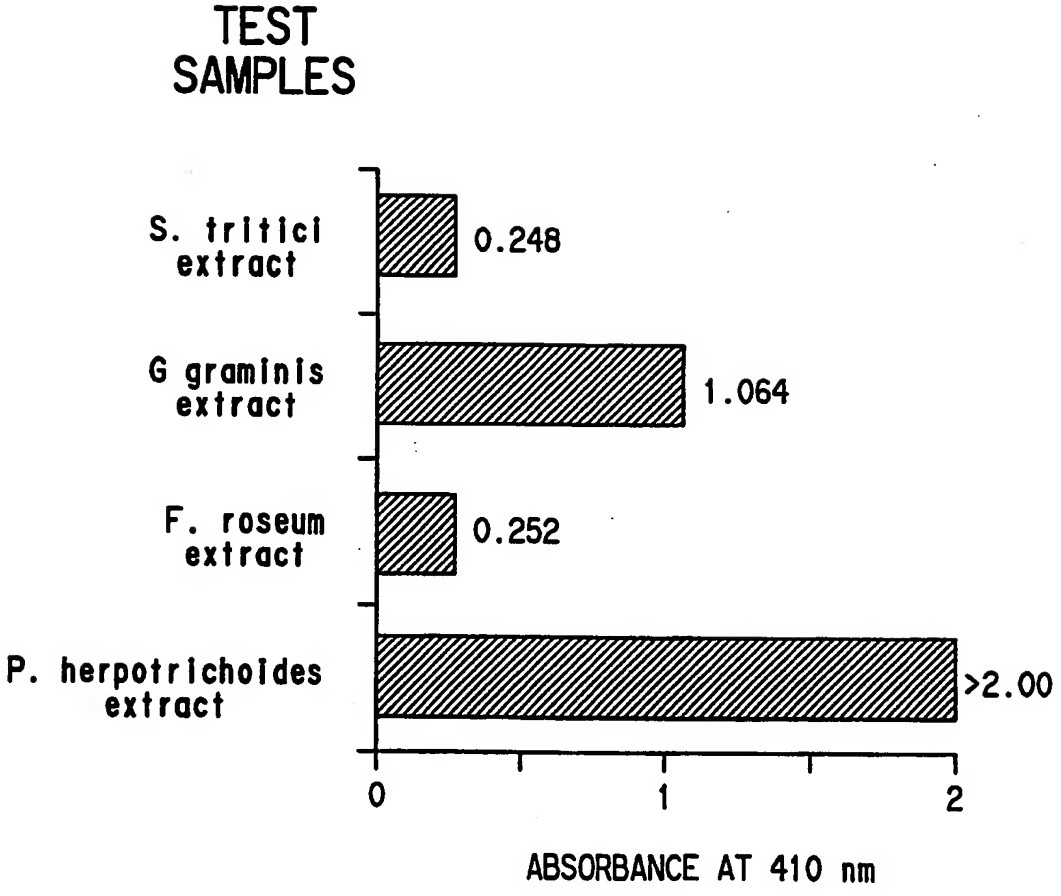

TEST SAMPLES
S. tritici extract
0.248
G graminis extract
1.064
F. roseum extract
0.252
P. herpotrichoides extract
>2.00
0
1
2
ABSORBANCE AT 410 nm

FIG.4

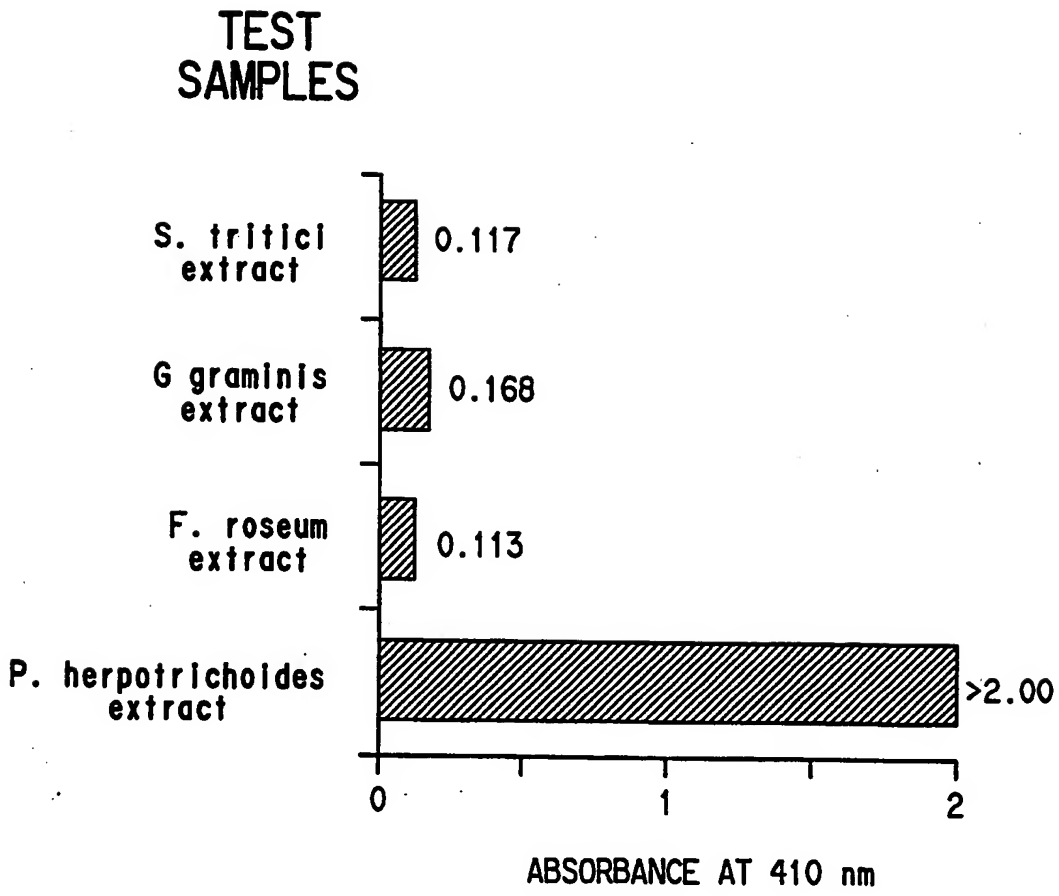

TEST SAMPLES
S. tritici extract
0.117
G graminis extract
0.168
F. roseum extract
0.113
P. herpotrichoides extract
>2.00
0
1
2
ABSORBANCE AT 410 nm

FIG.5

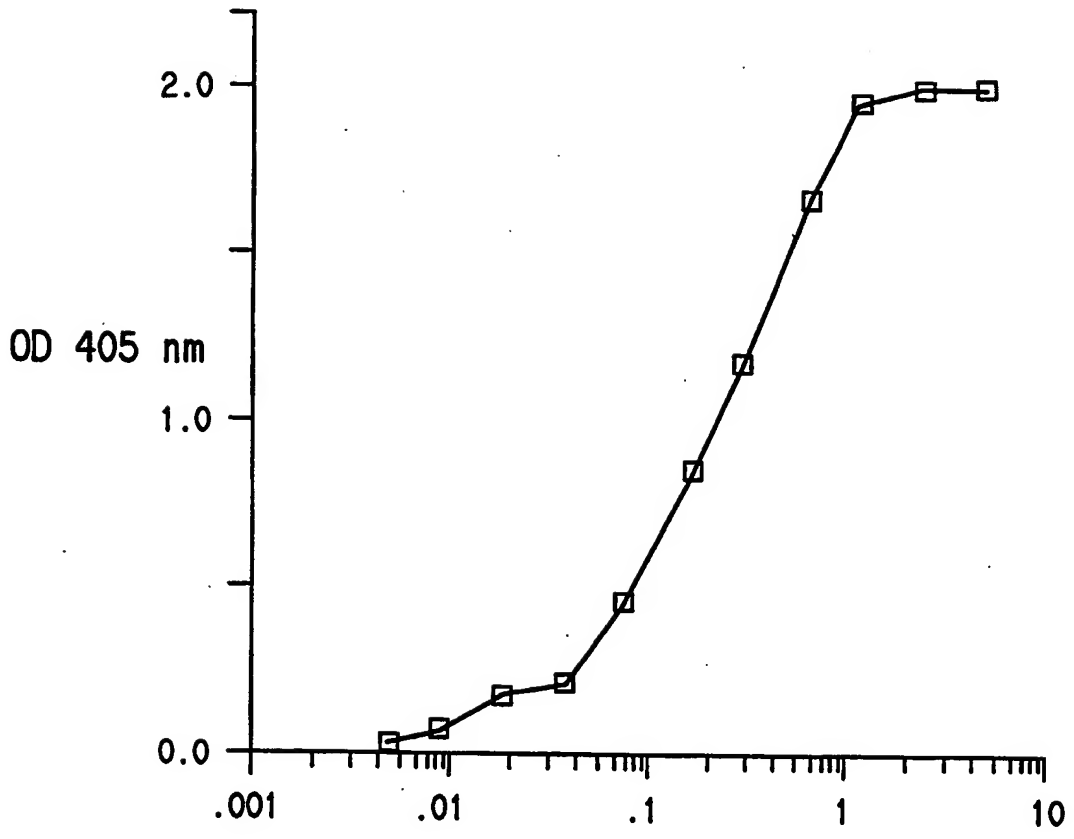
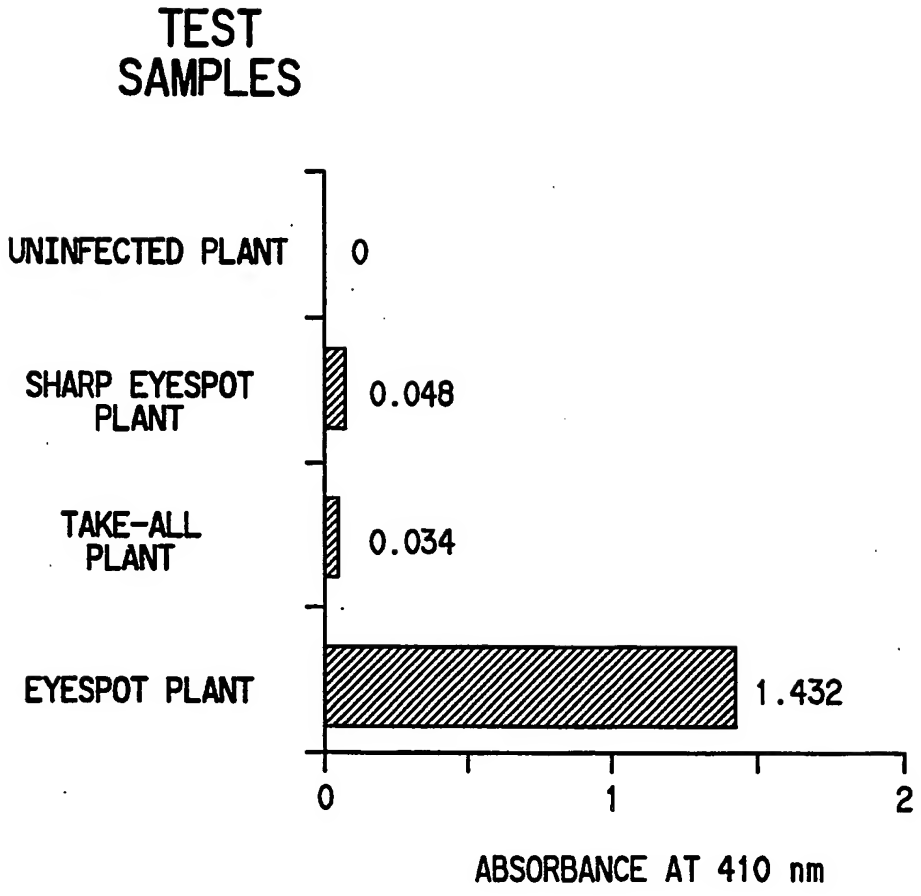


FIG.6


TEST SAMPLES
UNINFECTED PLANT
0
SHARP EYESPOT PLANT
0.048
TAKE-ALL PLANT
0.034
EYESPOT PLANT
1.432
0
1
2
ABSORBANCE AT 410 nm

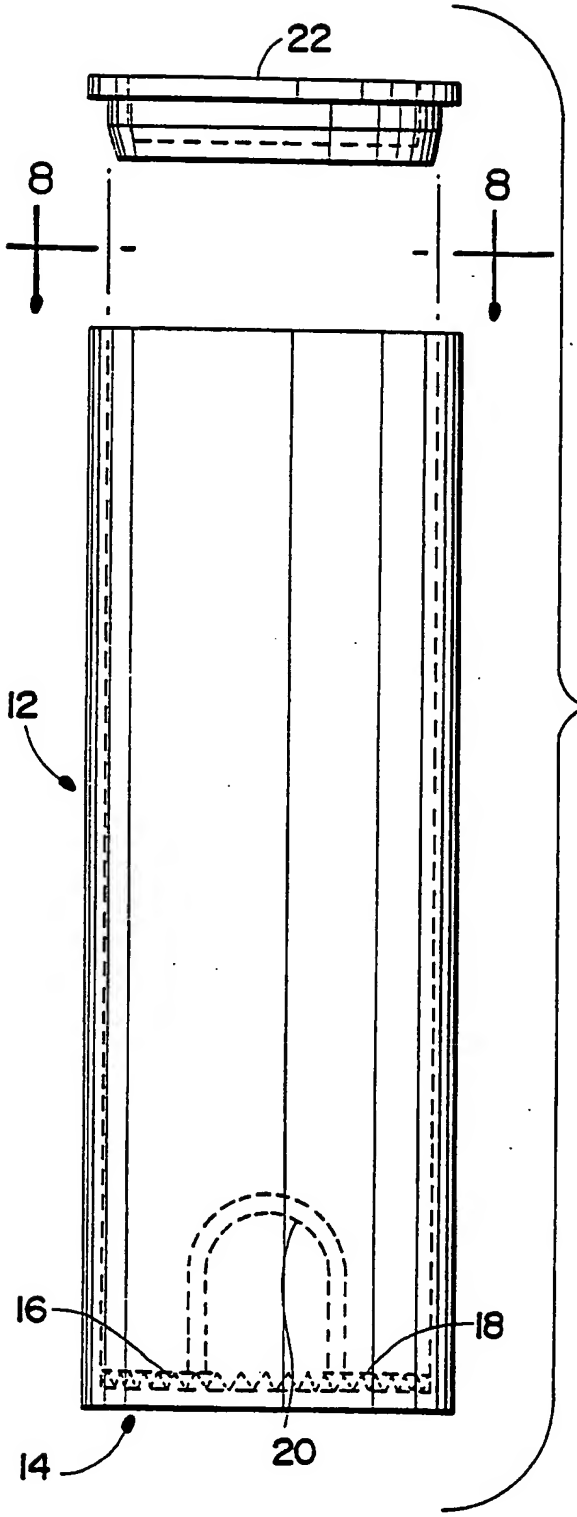


FIG. 7

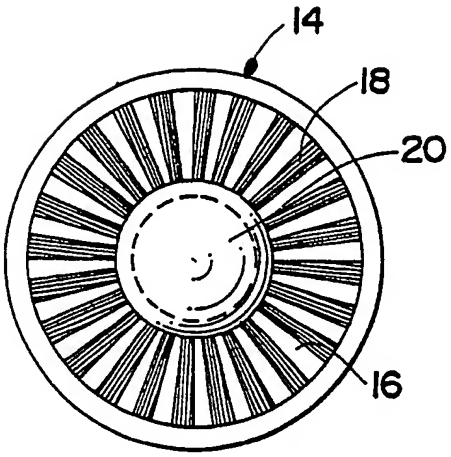


FIG. 8

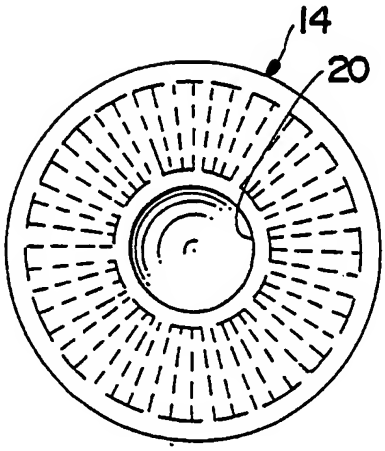


FIG. 9

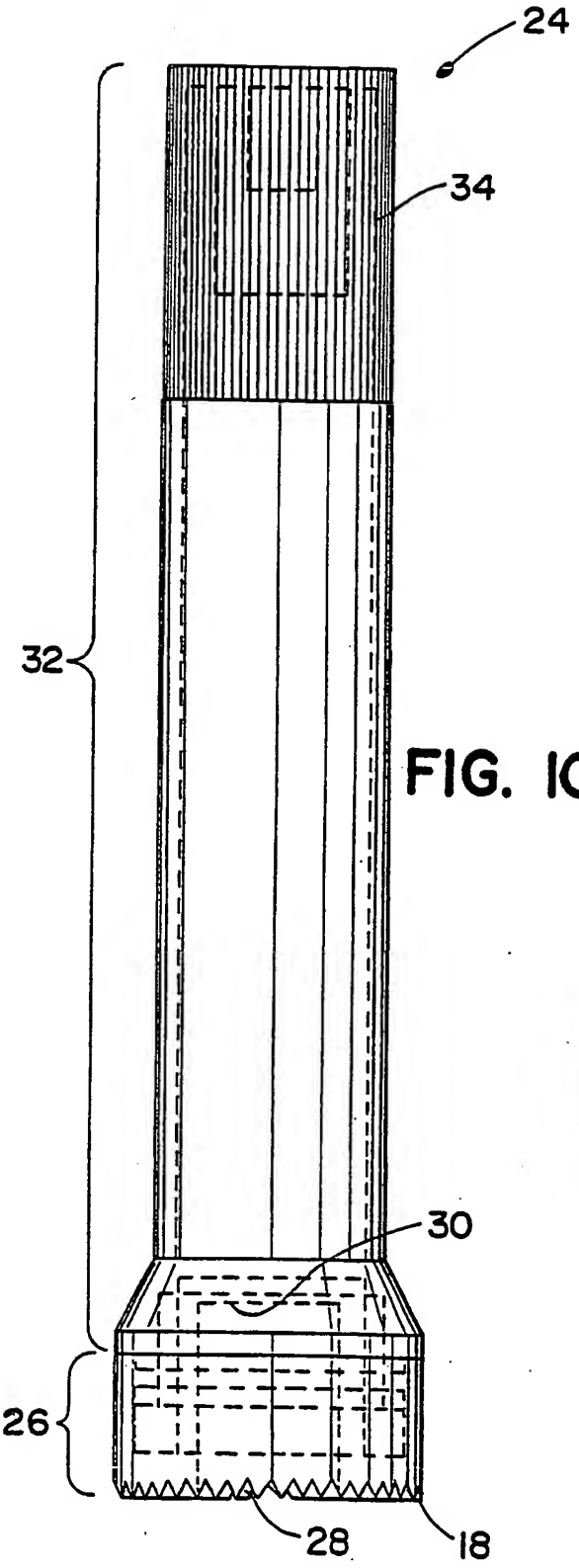


FIG. 10

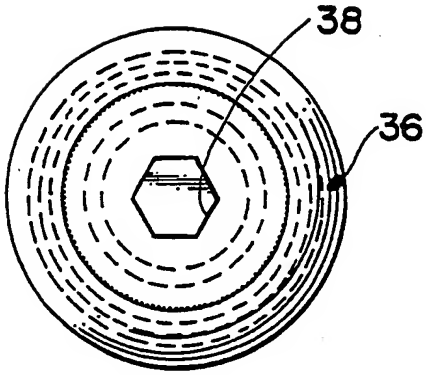


FIG. 11

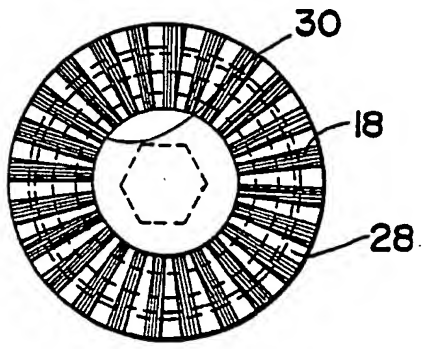


FIG. 12

FIG. 13

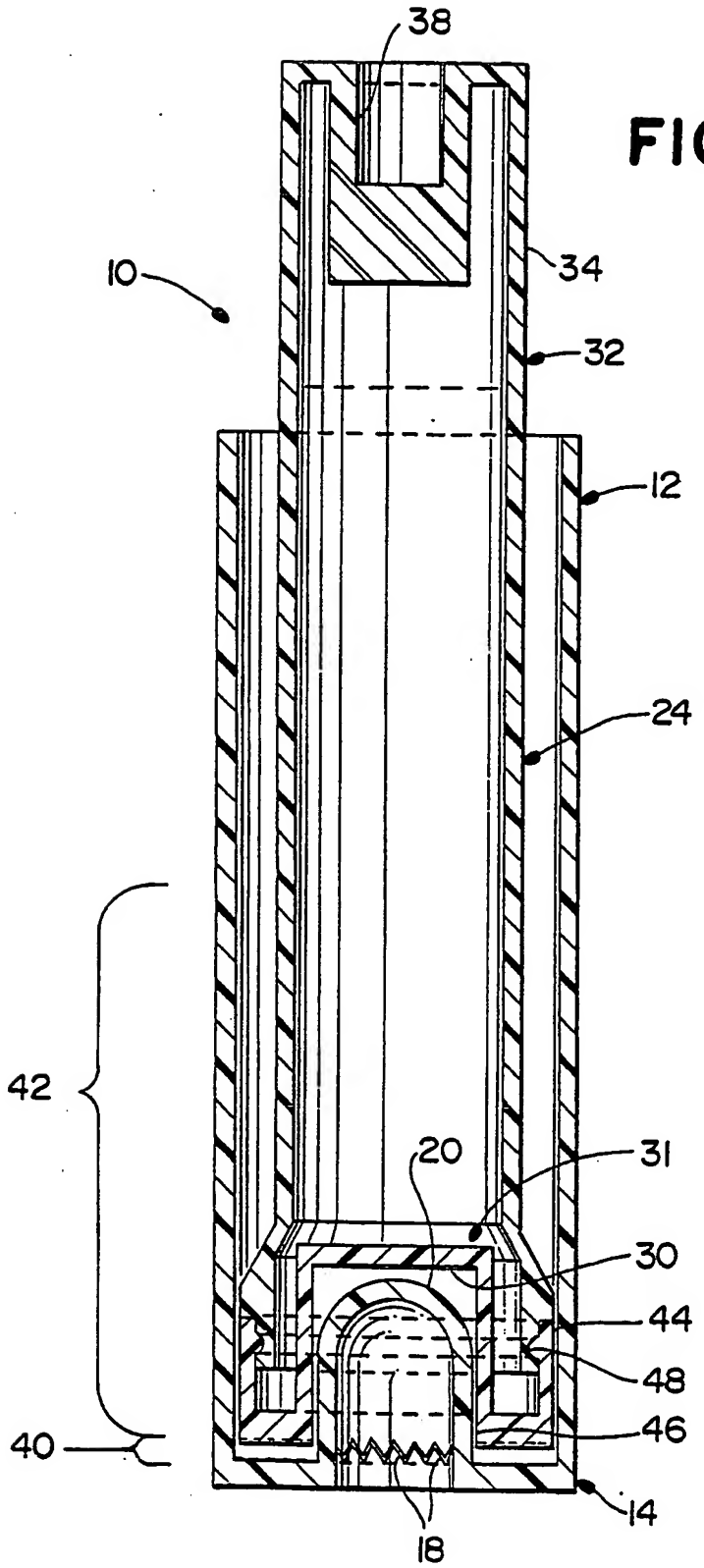


FIG. 14